[DESCRIPTION]

E.COLI MUTANT CONTAINING MUTANT GENES RELATED WITH TRYPTOPHAN BIOSYNTHESIS AND PRODUCTION METHOD OF TRYPTOPHAN BY USING THE SAME

[Technical Field]

The present invention relates to a Tryptophan-producing *E.coli* mutant strain CJ285 (KCCM-10534) containing single or multi mutant genes related with Tryptophan biosynthesis and production method of Tryptophan using the same. More particularly, N-methyl-N'-nitro-N-nitrosoguanidine (hereinafter it will be referred to as NTG) is processed repeatedly, and base sequences and amino acid sequences of genes originated from the Tryptophan-producing mutant gene CJ285, such as *aroF* and *aroG* for encoding isoenzyme of DAHP synthase that is resistant to Tryptophan Hydroxamate (hereinafter it will be referred to as THX), the Tryptophan analog, *trpR* for regulating *trp, aroH, mtr, trpR*, and *aroL* operon related with Tryptophan biosynthesis, and *tyrR* protein for regulating *aroF-tyrA, aroG*, and *aroP* operon are known. A mutant strain containing the above gene(s) is then fermented in a medium containing glucose in order to produce L-tryptophan.

[Background Art]

Tryptophan is one of essential amino acids, and has been broadly used in diverse fields including feed additives, medical substances such as sleeping draught or tranquilizer or Ringer's solution, and health food substances. Typical production methods of Tryptophan are chemical synthesis, enzyme reaction, and fermentation using microorganisms. In case of the chemical synthesis, the production takes place in a high temperature and high pressure space, and because D-tryptophan and L-tryptophan are produced together an additional refining process is required to obtain desired tryptophan. In case of the enzyme reaction such as the Japanese patent to Matsui Doatsui (Korean Patent Publication No. 90-005773), indole and serine used as

substrates for the reaction are very expensive and the enzyme itself is not safe.

On the other hand, the fermentation using microorganisms involve auxotrophic strains and regulatory mutant strains of diverse microorganisms such as *E.coli* and *Corynebacterium*. Rapid technical advances in gene recombination in 1980's have provided much information on metabolism and control mechanism thereof. Many researchers had remarkable successes to develop superior recombinant strains through gene manipulation, and to improve productivity (Matsui *et al*, 1988). Also, in Korea, a number of Tryptophan production techniques related with the direct fermentation were disclosed either by using Tryptophan-resistant or auxotrophic mutant strains (Korean Patent Publication Nos. 87-1813, 90-8251, and 92-7405) or recombined strains (Korean Patent Publication Nos. 90-5772 and 91-5627). Mainly these Tryptophan analog resistant strains were to overcome feedback inhibition of enzymes during the Tryptophan biosynthesis, and the recombinant strains were also used for cloning enzymes during the Tryptophan biosynthesis. In fact, the studies have made a remarkable success. For instance, the biggest merit of the traditional L-tryptophan production using an artificial mutant of *E.coli* was that inexpensive cultivation substrates were used to product the L-tryptophan. However, the productivity or the Tryptophan yield was extremely low. Therefore, to maximize the Tryptophan yield through the gene recombination, there exists a need to secure an artificial mutant which is excellent as a parent strain and obtain genes whose regulations are released.

Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to identify base sequences and amino acid sequences of a mutant gene for encoding *aroF* and *aroG,* which are enzymes for use in the synthesis of 3-deoxyarabionohep-tulosonate 7-phosphate (hereinafter it will be referred to as DAHP), the first precursor of aromatic amino acids during the biosynthesis of Tryptophan originated from an *E.coli* mutant strain CJ285, out of phosphoenolpyruvate and Erythorse 4-phosphate, and *trpR* and *tyrR* for regulation transcription of genes related with the Tryptophan synthesis.

It is another object of the present invention to provide an L-tryptophan

producing *E.coli* mutant strain that contains single or multi mutant genes described above.

It is still another object of the present invention to provide a production method of L-tryptophan with high concentration and high yield by cultivating the mutant directly in a fermentation medium containing glucose.

[Description of the Drawings]

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 illustrates a mutant gene, wherein CCT among the internal sequence of *aroF* gene is mutated to **[TCT]** and as a result thereof, the 280th amino acid Proline is changed to Serine;

Fig. 2 illustrates a mutant gene, wherein T base in the promoter region of *aroG* gene is mutated to **[C]** base, GTG in the internal sequence of the gene to **[GCG]**, and TGC to **[CGC]** and as a result thereof, the 57th amino acid Valine is changed to Alanine and the 61st amino acid Cysteine is changed to Arginine, respectively;

Fig. 3 illustrates a mutant gene, wherein the 704th G base in the internal sequence of *trpR* gene is deleted and as a result thereof, the frame during the protein translation is changed and 23 amino acids with respect to wild-type gene **[cgattgattttgtaggcctgataagacgtggcgcatcaggcatcgtgcaccgaatgccggatgcggcgtga]** are added; and

Fig. 4 illustrates a mutant gene, wherein GGC in the internal sequence of *tyrR* gene is mutated to **[GAC]** and CTG to **[CTA]** and as a result thereof, the 25th amino acid Glycine is changed to Aspartate and the 86th amino acid Leucine is changed to a nonsense mutation.

[Disclosure]

[Technical Problem]

In accordance with an aspect of the present invention, the above and other objects can be accomplished by the provision of an *E.coli* mutant strain containing mutant genes related with Tryptophan biosynthesis, in which NTG is processed repeatedly in *E.coli* CJ181 (KFCC 10902), the tryptophan-producing parent strain, to cause mutation therein and the mutant (CJ285) is made resistant to THX, the Tryptophan analog, whereby Tryptophan production of the mutant strain can be improved markedly compared to the parent strain. Also, to analyze DNA base sequence and amino acid sequence, genes for encoding *aroF* and *aroG*, the enzymes for synthesizing the first precursor DAHP of the aromatic amino acid during the Tryptophan biosynthesis, *trpR* protein for regulating *trp, aroH, mtr, trpR,* and *aroL* operon related with the Tryptophan biosynthesis, and *tyrR* protein for regulating *aroF-tyrA, aroG, aroP* operon are cloned, and the DNA base sequences is compared with the base sequences of wild-type genes. In this manner, it becomes possible to locate gene mutation. Later, CJ285 strain containing at least one of *aroF, aroG, trpR* and *tyrR* is cultivated directly in the fermentation medium containing glucose. Compared to the parent strain, the CJ285 produced much more Tryptophan.

That is to say, the *E.coli* CJ285 strain serves to maximize Tryptophan yield through the gene recombination technique and is a novel strain that has never disclosed.

[Technical Solution]

The present invention provides a production method of L-tryptophan wherein the method includes the steps of: amplifying primer of genes through the Polymerase Chain Reaction (PCR), the genes encoding enzymes involved in the synthesis of 3-deoxyarabionohep-tulosonate 7-phosphate (DAHP), *trpR* protein for regulating *trp, aroH, mtr, trpR,* and *aroL* operon related with Tryptophan biosynthesis, and *tyrR* protein for regulating *aroF-tyrA, aroG*, and *aroP* operon, cloning the genes by pCR2.1-TOPO vector to search plasmid clones that react with a band of expected size;

determining base sequences of *aroF, aroG, trpR,* and *tyrR* genes based on the bidirectional base sequence analysis employing the plasmid clone containing the above-described four genes as a template, determining amino acid sequences from the base sequences, and comparing the base sequences of the genes with the base sequences of wild-type genes to locate mutation; and fermenting an *E.coli* mutant strain CJ285 containing one or more of mutant genes *aroF, aroG, trpR,* and *tyrR* in a fermentation medium containing glucose and thereby, producing L-tryptophan.

The gene manipulation used in the present invention conforms to the Molecular Cloning Laboratory Manual (T. Maniatis E.F., Flitch, J. Sambrook).

A tryptophan-producing parent strain *E.coli* CJ181 (KFCC 10902) was cultivated at constant temperature for five days in a plate minimal medium containing 0.3g/l of THX, the Tryptophan analog. To increase growth rate and release the sensitivity of the CJ181 to THX, 500μg/ml of NTG, the mutation-causing substance, was added into the medium. Any strains grown in the minimal medium containing 0.3g/l of THX were selected first, and these selected strains were cultivated again in a minimal medium containing 0.5g/l of THX. Finally, a highly THX-resistant strain was selected and named CJ285. The ingredients of the minimal medium are shown in Table 1 below, and 100mg/l of auxotrophic amino acid was added to the medium, respectively.

[Table 1]

Composition of *E.coli* minimal medium (M9 medium)

Glucose minimal medium (M9 medium)	
Ingredient	Content (g/l)
Glucose	2
$NaHPO_4$	6
KH_2PO_4	3
NaCl	0.5
NH_4Cl	1
$MgSO_4$	0.5
$CaCl_2$	0.01

Tyrosine	0.1
pH 7.0	

The mutant strain CJ285 of the present invention went through 12-hour shaking culture in LB medium at 37°C. The LB medium (pH = 7.4) contained 1% of Bacto-Trypton, 0.5% of Bacto-yeast extract, and 1% of NaCl. Out of the medium was collected a mycobiont and a chromosome DNA was obtained by means of the Quiagen chromosomal DNA isolation kit. Thusly obtained chromosome DNA was immersed in ethanol and dried to be purified. This purified chromosome DNA, being as a template, went through the PCR. Approximately 1.3kb, 2kb, 530bp, and 1.9kb of *aroF, aroG, trpR*, and *tyrR* mutant genes were separated, respectively, from 1% of agarose gel by means of the Quiagen gel extraction kit, and the gene fragments were purified to use as genetic resources for cloning. These mutant gene fragments originated from the CJ285 strain were cloned to pCR2.1-TOTO vector by means of the TOPO cloning kit (manufactured by Invitrogen Company) and as a result thereof, a clone containing the gene was identified.

To determined the base sequence and the amino acid sequence of the gene for encoding *aroF, aroG, trpR*, and *tyrR* proteins, the plasmid containing mutant gene(s) was isolated and purified, and the entire gene sequence including the sequence after the promoter, genetic code region, protein synthesis termination codon was determined. In order to determine the DNA base sequence of the genes of the present invention, the previously isolated, purified plasmid DNA was mixed with a sequencing primer and a polymerase, and amplified through the PCR. Thusly amplified plasmid DNA was immersed in ethanol to be purified and mixed with Hi-Di solution. As a result, the plasmid DNA was transformed to a dsDNA containing single strand, and DNA base sequence analysis was proceeded by means of the base sequence analyzer ABI 3100 (manufactured by Applied Biosystem). The DNA base sequence analysis was performed on the basis of the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search program in the U.S. NCBI (National Center for Biotechnology Information) website

and the Tools PROGRAM (http://us.expasy.org/tools/dna.html) in the ExPasy website. Thusly determined gene base sequence was then compared with the base sequence of a wild-type gene to find out if any mutation occurred, and the mutant amino acid was identified through the translation.

The CJ285 mutant strain containing at least one of the mutant genes *aroF, aroG, trpR,* and *tyrR* was fermented directly in the fermentation medium containing glucose to produce L-tryptophan. More specifically, the mutant strain was cultivated under aerobic condition (flask shaking at 200-300rpm or fermenter at 400-1000rpm, and the amount of air current = 0.5-1.5vvm), fermentation temperature = 30°C and pH = 6.0 ~ 8.0, and the resulting L-tryptophan was accumulated into the culture medium. In case of using the flask, the mutant strain was cultivated at 30°C and 220rpm for 48-60 hours, and the resulting L-tryptophan was accumulated in the culture medium. In case of using the fermenter, fed batch cultivation is used. Thus, glucose was additionally supplied several times to produce L-tryptophan. The ingredients of the fermentation medium are listed in Table 2 below.

[Table 2]

Composition of fermentation medium

Fermentation medium in Erlenmeyer flask		Fermentation medium in 5L fermenter	
Ingredient	Content (g/l)	Ingredient	Content (g/l)
Glucose	60	Glucose	63.16
Yeast extract	2.5	Yeast extract	4
KH_2PO_4	2	KH_2PO_4	1.5
$MgSO_4 \bullet 7H_2O$	1	Citric acid	1.4
$(NH_4)_2SO_4$	20	$MgSO_4 \bullet 7H_2O$	2
Sodium citrate	5	$(NH_4)_2SO_4$	7
NaCl	1	Tyrosine	0.8
Tyrosine	0.1	Fumaric acid	1
$CaCO_3$	40	$CaCl_2$	0.5

To find out growth rate of the mycobiont of the culture medium, absorbance

was measured at 600nm. Also, the sugar analysis was made based on Bertrand method. Meanwhile, the quantity of L-tryptophan was analyzed by means of HPLC (High Performance Liquid Chromatography).

Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

[Advantageous Effects]

According to the present invention, the novel *E.coli* mutant strain CJ285 (KCCM-10534) is developed by processing NTG repeatedly in *E.coli* CJ181, and resistant to Tryptophan Hydroxamate, the Tryptophan analog. By analyzing DNA base sequence and amino acid of mutant genes *aroF, aroG, trpR*, and *tyrR* related with Tryptophan biosynthesis, a significant mutation can be identified and a proper mutant gene for use in the recombined strain development can be obtained. In addition, compared to the parent strain CJ181, its mutant strain CJ285 containing at least one of the mutant genes is capable of producing more Tryptophan (about 10% more). Therefore, CJ285 of the present invention can be very advantageously used as a proper mother strain for the recombined strain development and for the amino acid fermentation industry and pharmaceutical manufacture.

[Mode for carrying out the Invention]

Example 1: Selection of THX-resistant mutant strain CJ285

Tryptophan-producing parent strain *E.coli* CJ181 (KFCC 10902) went through 12-hour shaking culture in LB medium at 37°C, and was rinsed twice in sterilized saline solution. Here, the LB medium (pH = 7.4) contained 1% of Bacto-Trypton, 0.5% of Bacto-yeast extract, and 1% of NaCl. The CJ181 was diluted in 0.1M sodium citrate buffer solution (pH = 5.5) until the final OD = 1.0. The CJ181 was cultivated in the minimal medium containing 0.3g/l of THX (please refer to Table 1) for five days. In order to increase growth rate and make the THX-resistant CJ181, 500μg/ml of NTG,

the mutation-causing substance, was added into the medium. The solution was placed in a 37°C thermostatic bath for the reaction for 30 minutes, and was rinsed in 0.1M phosphate buffer solution (pH = 7.0) three times. Then the CJ181 was cultivated in a minimal medium containing 0.5g/l of THX (please refer to Table 1) for five days and as a result thereof, approximately 100 colonies were obtained. Thusly obtained mutant strains and the original parent strain were subject to Tryptophan fermentation test in a flask. In result, *E.coli* CJ285 featuring superior tryptophan production capacity to the original *E.coli* parent strain CJ181 could be selected. The best strain for producing L-tryptophan was isolated from the strains, and placed in an Erlenmeyer flask. After conducting the tryptophan production test on the strain in the flask, and the fermentation experiment in the 5L fermenter was performed as described in Example 6 below.

[Table 3]

Experiment result of newly developed artificial mutant strains in flask

E.coli	Mycobiant (OD_{600})	L-tryptophan (g/l)
CJ181	30	7.1
CJ285	28	7.9

As seen in Table 3, the concentration of L-tryptophan produced from the *E.coli* mutant strain CJ285 was higher than that of the original parent strain *E.coli* KFCC 10902. The CJ285 was deposited with the KCCM (Korean Culture Center of Microorganisms) on November 28, 2003, and given the number KCCM-10534.

Example 2: *aroF* gene cloning of mutant strain CJ285 and sequence analysis

To amplify *aroF* gene through the PCR using a chromosome DNA isolated from the CJ285 as a template, the following primers (21-mers) were used. 5'-GTATTTACCCCGTTATTGTC-3' was used as a sense primer, and 5'-CACTTCAGCAACCAGTTCCAG-3' was used as an anti-sense primer. For the PCR about 30ng of genomic DNA of the CJ285 and 25pmol of each primer were added to

Accupower PCR HL-Premix containing DNA polymerase, dNTPs and reaction buffer until the final concentration becomes 20μℓ. The PCR program was executed 25 times. It started at 94°C for five minutes and then 35 seconds, at 55°C for 40 seconds, and at 72°C for 90 seconds. Lastly, the last extension was performed for seven minutes at 72°C. Its result was then checked through 1% agarose gel electrophoresis.

To clone the gene fragment to pCR2.1-TOPO vector, about 1.3kb (which corresponds to the size of *aroF* mutant gene) gene fragments were isolated from the 1% agarose gel by means of the Quiagen gel extraction kit. Thusly obtained gene fragments were then purified and used as genetic resources for cloning. By using the TOPO cloning kit (manufactured by Invitrogen Company) the mutant gene fragments obtained form the CJ285 strain were mixed with pCR2.1-TOPO vector solution at the ratio of 1:4. Later, 1μℓ of saline solution was added to the mixture and the reaction was continued at room temperature for 20 minutes. The reaction solution was mixed with 40μℓ of TOP10 competent cell included in the kit and sit in the ice for 20 minutes. Afterwards, thermal shock was applied for 30 seconds at 42°C and the solution was placed back to the ice immediately for 2 minutes. 250μℓ of SOC medium was added thereto, and the mutant genes were cultivated at 37°C for 1 hour. 100μℓ of culture medium was smeared over LB agar medium containing 50μg/ml of Ampicillin, and the mutant genes were cultivated therein for about 12 hours at 37°C. Only white colonies were selected and cultivated again for about 12 hours in the LB liquid medium containing 50μg/ml of Ampicillin. Plasmid was isolated therefrom and treated with *Eco*RI restriction enzyme for 2 hours and developed by 1% agarose gel electrophoresis. By using an UV illuminator, a clone containing the mutant gene(s) was identified.

To determine DNA base sequence of the genes, plasmid was isolated from the

previously identified clone and purified. Thusly isolated, purified plasmid was mixed with 2pmol of sequence analysis primer that can be combined with *aroF* gene through a complementary hydrogen bond, 2μℓ of Big dye containing polymerase, and 1μℓ of plasmid DNA (about 200ng). Then, the PCR was executed 25 times, first at 96°C for 30 seconds, at 50°C for 15 seconds, and at 60°C for four minutes. The plasmid DNA was immersed in ethanol to be purified and mixed with 10μℓ of Hi-Di solution. As a result, the plasmid DNA was transformed to a dsDNA containing single strand, and DNA base sequence analysis was proceeded by means of the base sequence analyzer ABI 3100 (manufactured by Applied Biosystem). The DNA base sequence analysis was performed on the basis of the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search program in the U.S. NCBI (National Center for Biotechnology Information) website and the Tools PROGRAM (http://us.expasy.org/tools/dna.html) in the ExPasy website. Thusly determined gene base sequence was then compared with the base sequence of a wild-type gene to find out if any mutation occurred, and the mutant amino acid was identified through the translation.

Example 3: *aroG* gene cloning of mutant strain CJ285 and sequence analysis

To amplify *aroG* gene through the PCR using a chromosome DNA isolated from the CJ285 as a template, the following primers (21-mers) were used. 5'-GTATTTACCCCGTTATTGTC-3' was used as a sense primer, and 5'-ACTCCGCCGGAAGTGACTAA-3' was used as an anti-sense primer. For the PCR about 30ng of genomic DNA of the CJ285 and 25pmol of each primer were added to Accupower PCR HL-Premix containing DNA polymerase, dNTPs and reaction buffer until the final concentration becomes 20μℓ. The PCR program was executed 25 times. It started at 94°C for five minutes and then 35 seconds, at 55°C for 40 seconds, and at 72°C for two minutes and 20 seconds. Lastly, the last extension was performed for seven minutes at 72°C. Its result was then checked through 1% agarose gel

electrophoresis.

To clone the gene fragment to pCR2.1-TOPO vector, about 2kb (which corresponds to the size of *aroG* mutant gene) gene fragments were isolated by means of the Quiagen gel extraction kit. Thusly obtained gene fragments were then purified and used as genetic resources for cloning. The experiment procedure from this point and the base sequence analysis following the determination of base sequence are identical with those in Example 2.

Example 4: *trpR* gene cloning of mutant strain CJ285 and sequence analysis

To amplify *trpR* gene through the PCR using a chromosome DNA isolated from the CJ285 as a template, the following primers (21-mers) were used. 5'-CGCCACGGAATGGGGACGTCG-3' was used as a sense primer, and 5'-CCGCGTCTTATCATGCCTACC-3' was used as an anti-sense primer. For the PCR about 30ng of genomic DNA of the CJ285 and 25pmol of each primer were added to Accupower PCR HL-Premix containing DNA polymerase, dNTPs and reaction buffer until the final concentration becomes 20μℓ. The PCR program was executed 25 times. It started at 94°C for five minutes and then 1 minute, at 60°C for 30 seconds, and at 72°C for 1 minute. Lastly, the last extension was performed for seven minutes at 72°C. Its result was then checked through 1% agarose gel electrophoresis.

To clone the gene fragment to pCR2.1-TOPO vector, about 530bp (which corresponds to the size of *trpR* mutant gene) gene fragments were isolated by means of the Quiagen gel extraction kit. Thusly obtained gene fragments were then purified and used as genetic resources for cloning. The experiment procedure from this point and the base sequence analysis following the determination of base sequence are identical with those in Example 2.

Example 5: *tyrR* gene cloning of mutant strain CJ285 and sequence analysis

To amplify *tyrR* gene through the PCR using a chromosome DNA isolated

from the CJ285 as a template, the following primers (21-mers) were used. 5'-GGATTGACGATGACAAACCT-3' was used as a sense primer, and 5'-CTGGTGGATGAAATCACCAC -3' was used as an anti-sense primer. For the PCR about 30ng of genomic DNA of the CJ285 and 25pmol of each primer were added to Accupower PCR HL-Premix containing DNA polymerase, dNTPs and reaction buffer until the final concentration becomes 20μℓ. The PCR program was executed 25 times. It started at 94°C for five minutes and then 1 minute, at 53°C for 30 seconds, and at 72°C for two minutes and 20 seconds. Lastly, the last extension was performed for seven minutes at 72°C. Its result was then checked through 1% agarose gel electrophoresis.

To clone the gene fragment to pCR2.1-TOPO vector, about 1.9kb (which corresponds to the size of *tyrR* mutant gene) gene fragments were isolated by means of the Quiagen gel extract kit. Thusly obtained gene fragments were then purified and used as genetic resources for cloning. The experiment procedure from this point and the base sequence analysis following the determination of base sequence are identical with those in Example 2.

Example 6: Fermentation of mutant strain CJ285 in 5L fermenter

E.coli CJ285 containing at least one of the mutant genes with the base sequence disclosed in the Examples 2 to 5, and the parent strain CJ181 (KFCC 10902) were fed batch cultivated in a 5L fermenter (fermentation temperature = 30°C, culture pH = 6.9 – 7.1 (pH can be controlled by ammonia water), the amount of air current = 0.5 – 1.0vvm, and stirring speed = 500 – 700rpm). It turned out the fermentation concentration of the CJ285 was 28.2g/l, and that of the parent strain CJ181 was 25.1g/l. Therefore, the fermentation time of the *E.coli* CJ285 was reduced slightly, resulting in L-tryptophan productivity increase by about 10% per hour.

[Table 4]

Fermentation of CJ285 mutant strain in 5L fermenter

Name of strain	Cultivation time (hr)	Total sugar (g/l)	Total amount of accumulated T-tryptophan
CJ181	63	243.5	25.1
CJ285	61	243.5	28.2